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GRANT NUMBER DAMD17-95-1-5003

TITLE: Identification of Novel Candidate Tumor Suppressor Genes Using C. elegans as a Model

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REPORT DATE: November 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	November 1996	Annual (1 Nov 95 - 31 Oct 96)

4. TITLE AND SUBTITLE
Identification of Novel Candidate Tumor Suppressor Genes Using C. elegans as a Model

5. FUNDING NUMBERS
DAMD17-95-1-5003

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
California Institute of Technology
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Molecular genetic analysis of the model organism *Caenorhabditis elegans* was used to identify and study mechanisms of action of negative regulators of tyrosine kinase/*ras* mediated signal transduction that are candidate tumor suppressors. A homolog of proto oncogene *cbl, sli-1,* inhibits Ras activation by epidermal growth factor receptor homolog LET-23. Functional domains of SLI-1 were analyzed in transgenic nematodes. The *rok-1* gene was also shown to regulate Ras activation. The *rok-1* locus was cloned and shown to encode a novel tyrosine kinase with protein-protein interaction domains. ROK-1 protein physically interacts with the adaptor protein SEM-5, and thus might exert its negative effect both by being recruited to the EGF-receptor signaling complex and by preventing SEM-5 from leading to ras activation. New screens for additional negative regulators have been initiated to find partners for the SLI-1 and ROK-1. Human homologs of ROK-1 will now be sought.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, Tumor Suppressors, Growth Factor Receptors, Model System, Molecular Cloning, Proto-Oncogenes, Genetic Analysis	26
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Paul W Stewart 11-25-96

PI - Signature Date

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Introduction

An important short-term goal of breast cancer research is to identify candidate genes for identifying pre cancerous cells and cancerous cells. Ideally, pre cancerous or cancerous cells could be characterized by a set of molecular markers. These markers would best be chosen from the set of genes altered during early stages of precancer formation. It is also crucial to understand the cellular regulatory pathways in which these genes act. *C. elegans* molecular genetics provides a facile model system with which to identify and to examine gene interactions *in vivo* [reviewed by Sternberg, 1993]. For example, it has been clearly shown that the *C. elegans* ras protein LET-60 acts downstream of EGF-receptor homolog LET-23 [Han & Sternberg, 1990; Aroian et al. 1990], that the adaptor protein SEM-5 acts between LET-23 and ras [Clark et al., 1992a; Katz, et al., 1996] and that the LIN-45 raf protein acts downstream of LET-60 ras [Han et al., 1993]. This universal signaling pathway is the target of many mutations contributing to oncogenesis in humans. Overexpression or activation by mutation of LIN-3, LET-23, LET-60 results in excessive vulval differentiation; thus activation of the homologous genes leads to cancer in humans and vulval differentiation in nematodes. Therefore, negative regulators of vulval differentiation defined by loss-of-function mutations that lead to excessive signaling are analogous to tumor suppressor loci.

Using the powerful genetics of *C. elegans*, we have identified five pathways of negative regulation of LET-23 mediated signaling have been identified. We are carrying out genetic screens that can identify additional genes involved in negative regulation, either in known pathways or in new ones. Our genetic studies have identified several negative regulators of LET-23-mediated signaling in *C. elegans* [reviewed by Sternberg, 1994]: SLI-1 [Jongeward et al., 1995; Yoon et al., 1995], UNC-101 [Lee et al., 1994], ROK-1 [see details below], LIN-15 [Ferguson & Horvitz, 1989; Huang et al., 1994]. In general, these negative regulators are redundant, such that elimination of any one has no effect on the normal signal transduction. In the absence of two regulators, excessive vulval differentiation occurs. These genetic properties are similar to the synergistic action of oncogenic mutations, where several mutations are necessary for a phenotypic change. However, such synthetic mutations are difficult to study except in genetically facile organisms such as *C. elegans.*

If we can identify many loci possibly involved in the analogous processes in *C. elegans*, and identify human homologs, this will help human geneticists in several ways. First, this will provide candidate genes for the positional cloning of tumor suppressor loci defined by human cancer genetics. Second, this will provide molecular probes with which to examine tumorous tissue for alterations. The sooner we can identify the many potential tumor suppressor loci, the more effectively that analysis of the role of tumor suppressor mutation in breast cancer initiation and progression can be assessed. Current technology easily allows parallel processing of samples, and is thus limited by the number of molecular probes. Lastly, we can link together tumor suppressor genes in functional pathways, much as we have been able to do for the LET-23-mediated pathway. If there are multiple pathways of tumor suppressor gene action, then we need to know how to intervene in each one.

One implication of multistep carcinogenesis is that synergism occurs between mutations. The genetics of the negative regulators that we have identified is analogous: mutation of SLI-1 or ROK-1 alone causes no defect, yet inactivation of both leads to increased signaling. The roles of such apparently redundant genes are difficult to study except in powerful genetic systems; *C. elegans* vulval differentiation provides such a system.

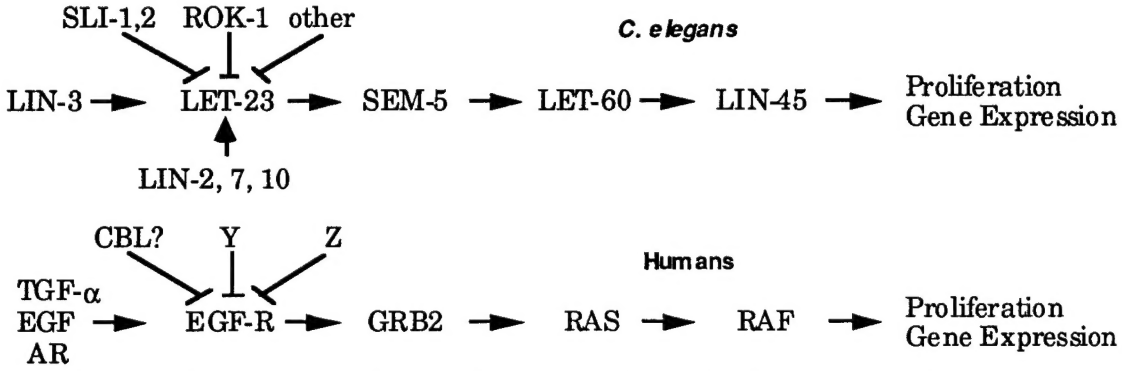


Figure 1. Pathway for the major EGF-R signaling pathway in humans and the analogous LET-23-mediated signaling pathway in *C. elegans*. Arrows indicated positive regulation; bars represent negative regulation. Other components are known.

The oncoprotein cbl has continued to be limited to signaling in many human cell types. Its physiological role remains elusive, and our molecular genetic studies of SLI-I are designed to help understand the function(s) of this new family of proteins. The roles of cytoplasmic tyrosine kinases in signaling and its regulation is another major area of research. We have discovered that *rok-1* encodes such a tyrosine kinase, and we have begun to examine its mechanism of action as well.

Progress

The goal of this project is to identify potential tumor suppressor genes and link them into functional pathways with each other, and with known proto-oncogenes and tumor suppressor genes. *C. elegans* vulval differentiation provides a facile model system with which to study EGF-receptor/*c-neu*-mediated signal transduction and its regulation. The *C. elegans sli-1, sli-2* and *rok-1* genes negatively regulate LET-23. We are using *C. elegans* molecular genetics to study and clone these genes in *C. elegans*, and will use molecular biology to clone their human homologs. The specific goals of the project are as follows.

1. **Analyze SLI-1 function** in *C. elegans* through molecular genetics.
2. **Molecularly clone *sli-2*.**
3. **Molecularly clone *rok-1*.**
4. **Identify and clone additional genes acting in concert with *sli-1*, *sli-2*, and *rok-1***
5. **Examine the functional interactions of *sli-1*, *sli-2*, *rok-1* in regulating other conserved signaling pathways.**
6. **Clone human *sli-2*, *rok-1*, and newly identified genes from human breast tissue libraries** to generate reagents with which to test the hypothesis that these are novel tumor suppressor loci.
7. **Test the functional homology of *c-cbl* and *sli-1*** by introducing the human cDNA into transgenic nematodes defective in *sli-1*.

In the following, progress on these goals is described.

1. Molecular genetics of *sli-1*

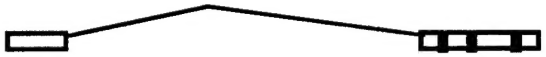
We identified the *sli-1* locus as a negative regulator of LET-23, the *C. elegans* homolog of EGF-R/c-neu/HER3/HER4, using extragenic suppressor analysis [Jongeward et al., 1995]. We cloned the *sli-1* locus by correlating genetic and physical maps and rescuing a *sli-1* mutant in transgenic nematodes [Yoon et al., 1995]. A 10.5 kilobase genomic fragment has the ability to provide all known functions of *sli-1* in transgenic animals, i.e., it rescues the suppression phenotype of *let-23*.

c-cbl, cbl-b and *sli-1* each contain a Ring Finger motif [$CX_2C_{9\text{-}27}CX_{1\text{-}3}HX_2CX_2CX_{6\text{-}48}CX_2C$] putative metal binding finger [Lovering et al., 1993]. The truncated, oncogenic form of *v-cbl* is missing the finger, as is the truncated form found in HUT78 T cell lymphoma cells [Blake & Langdon, 1992]. To examine structure/function relationships of *sli-1*, we have designed a minigene in which a tissue-general heat-inducible promoter/enhancer, *hsp-16*, directs transcription of a *sli-1* full length cDNA under the control of its own 5' control region, but found that this construct provides variable rescue in transgenic animals, likely because *sli-1* introns provide regulatory information. The *hsp16::sli-1* constructs provide strong rescuing activity by the sensitive vulval induction assay in which DNA is introduced into a *let-23(sy1); sli-1(sy143)* recipient (with *dpy-20* as a transformation marker; Without a transgene, the recipient strain displays 120% of wild-type vulval differentiation (Table 1A); introduction of the full length *sli-1* construct provides full *sli-1(+)* activity, reducing vulval differentiation to approximately 10% (Table 1B). Thus, in this assay, *sli-1(+)* function is indicated by low vulval differentiation; lack

of *sli-1* function is indicated by high vulval differentiation. We constructed a series of forms of *sli-1* (Table 1C-H). Deletion of a single proline-rich domain has a partial effect (Table 1E, I) or no effect (Table 1G). Deletion of the active proline-rich tail (Table 1C) curtails but does not eliminate *sli-1* activity. By constant deletion of the entire "catalytic" domain strongly reduces but apparently does not eliminate *sli-1* function (Table 1D). Last, deletion of the Ring Finger decreases but does not eliminate *sli-1* function (Table 1H). From these observations we conclude that both the Ring Finger (or catalytic domain) and the proline-rich region is important for *sli-1* activity in *vivo*. We will now test protein-protein interactions as we have started for ROK-1 (see below).

To test for dominant effects, we transformed each construct into a *sli-1* wild-type background *(dpy-20)* and scored vulval differentiation. We found no dominant effect. We will test some of these constructs in a more sensitive background, *(let-13(sy1)* before concluding that there is no dominant effect of *v-cbl*-like mutations in *sli-1*.

Table 1. *sli-1* Structure and Function. Individual larval hermaphrodites carrying the transgene are recognized by their non-Dpy phenotype and examined with Nomarski optics to determine the extent of vulval differentiation. A wild-type animal always has three VPCs generating vulval cells. *let-23(sy1)* reduces vulval differentiation to 0.3 VPCs. A *sli-1* mutation restores vulval differentiation to >3 VPCs. The gray box indicates the domain highly conserved between *sli-1, cbl* and *cbl-b*, a presumed "catalytic" domain. The hatched box indicates the ring finger motif. The black bars represent proline-rich putative SH3-binding domains. Deleted regions are indicated by their angled lines.

***sli-1* MINIGENE CONSTRUCTS INJECTED**	**PARENTAL GENOTYPE**	
	sy1 ; sy143	***+ ; +***
A. NO CONSTRUCTS	3.6 (120%) n=25	3.0 (100%) n>20
B. 	0.2 (8%) n=13 0.1 (3%) n=18	not determined
C. 	1.0 (33%) n=27 1.5 (50%) n=36	3.0 (100%) n=20 3.0 (100%) n=20
D. 	2.2 (73%) n=47 2.8 (93%) n=35	3.0 (100%) n=20 3.0 (100%) n=20
E. 	0.5 (17%) n=31 1.0 (33%) n=24	3.0 (100%) n=20 3.0 (100%) n=20
F. 	0.8 (27%) n=26 0.9 (30%) n=30	3.0 (100%) n=20 3.0 (100%) n=20
G. 	0.3 (10%) n=33 0.4 (13%) n=30	3.0 (100%) n=20 3.0 (100%) n=20
H. 	1.2 (40%) n=31 1.7 (57%) n=35	3.0 (100%) n=20 3.0 (100%) n=20

To test the hypothesis that *sli-1* mutations bypass the requirement for Ras, strains mutant in a series of *let-60 ras* alleles [Beitel et al., 1990] and *sli-1(sy143)* were constructed last year. The lethality but not the vulvalessness of *let-60* mutations were suppressed in the F1 generation. F2 homozygotes from homozygous *let-60* mothers all died, consistent without previous observations that *sli-1(sy143)* does not suppress complete loss-of-function *let-60* mutations for viability [Jongeward et al., 1995]. This observation indicates that during vulval induction, *sli-1* is regulating ras activation. A critical control for this *let-60* suppression experiment is to know that the *let-60* alleles used (*n2022*, *n2034*, *n2035*, and *n1876*) are not null. We constructed *trans* heterozygotes of these alleles, and the non-null allele *n2021*, which allows a test of whether a *let-60* allele is non-null. A wild-type allele in *trans* to *n2021* is wildtype, with 3.0 VPCs induced. A deletion of *let-60* null allele in *trans* to *n2021* had ~0.7 VPCs induced (Table 2). The alleles *n1876, n2022* and *n2035* clearly provide some *let-60* activity as vulval induction is >1 VPC in this assay. We conclude that *sli-1(1f)* is unable to suppress severe but not null alleles of *let-60* (Table 3).

We have now tested the interactions of *sli-1* with *sem-5* [Clark et al., 1992a]. The severe but non-null allele of *sem-5*, *n1619*, is strongly suppressed by *sli-1(sy194)*. M. Stern (Yale University) has recently sent us candidate null alleles of *sem-5* to test for suppression.

Table 2. Severe but non-null *let-60* alleles. N2 males were mated to *dpy-10/dpy-10; unc-24 let-60(X)/DnT1* hermaphrodites and non-Unc male cross progeny (*unc-24 let-60(X)/++)* crossed to *dpy-10; unc-24 let-60(n2021)/DnT1* hermaphrodites. The Unc-24 non-Dpy-10 cross progeny (*unc-24 let-60(X)/unc-24 let-60(n2021)* were scored. *syl27am* is a null allele ((Han and Sternberg, 1991); *sDf8* is a deletion of the *let-60* locus.

genotype	Vulval induction	
+/n2021	3.0	many
n2021/n2021	2.2	n=24
n2022/n2021	2.9	n=21
n2035/n2021	1.6	n=40
n1876/n2021	1.4	n=38
n2034/n2021	0.8	n=24
sy127am/n2021	0.8	n=23
sDf8/n2021	0.6	n=24

Table 3. Suppression of the inviability but not the vulvalessness of *let-60 ras* mutations by a *sli-1* mutation. The phenotypes of F1 homozygous *let-60* animals from heterozygous mothers were determined for four partially defective, recessive *let-60 ras* mutations (Beitel et al., 1990). *let-60* homozygotes were identified with a closely linked marker *unc-24*. For viability counts, the total progeny of 10 hermaphrodites of each genotype were counted (approx. 2000 hatched larvae per strain), and the number of Unc (*let-60/let-60*) adults compared to total progeny.

genotype	Viability		Vulval differentiation	
	sli-1(+)	*sli-1(-)*	*sli-1(+)*	*sli-1(-)*
+	100%	100%	3.0	3.0
let-60(n2022)	16	85	2.99	2.95
let-60(n1876)	14	100	0.0	0.052
let-60(n2034)	17	87	0.045	0.086
let-60(n2035)	22	89	0.016	0.034
sem-5(n1619)	--	--	0.25	3.03

2. *sli-2.* Yeast artificial clones have been prepared and awaiting injection.

3. Genetics and molecular cloning of *rok-1*

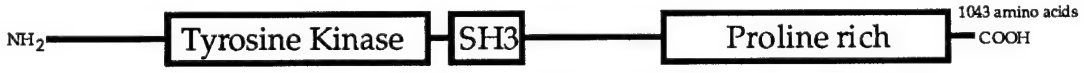
We identified the *rok-1* (*r*egulator *o*f *k*inase) locus (*IV*) of the nematode *C. elegans* in a genetic screen for new negative regulators of the vulval induction pathway. At 20°C, *rok-1(sy247)* causes essentially no phenotypes in an otherwise wild-type background. However, in combination with mutations in other negative regulator genes, *unc-101*, and *sli-1*, it causes pleiotropic effects including excessive vulval differentiation larval lethality and abnormal spicules. Animals defective in all three negative regulatory genes have a greater extent of vulval differentiation than any single or double mutants. *rok-1(sy247)* causes a slight temperature-sensitive gonad-dependent excessive vulval differentiation. We propose that *rok- 1* defines a fifth negative regulatory activity acting on the vulval induction pathway. *rok-1* differs in other negative regulators in that it appears to be exclusively on stimulated activity of the signaling pathway. The apparent redundancy differs with respect to different phenotypes. Last year we identified an 11.5 kb genomic clone that complements a *rok-1* mutation in transgenic nematodes. We have now analyzed the sequence and to some extent the mechanism of action of *rok-1*.

Genetic analyses have identified several genes which negatively regulate LET-23 activity in *Caenorhabditis elegans*. LET-23 is a member of the *neu*/epidermal growth factor receptor family of receptor tyrosine kinases which mediates intercellular signalling during development of *C. elegans*. Two of these negative regulators have been cloned; *unc-101*, which encodes the clatherin associated protein AP47, and *sli-1*, which encodes a protein homologous to the c-cbl proto-oncogene product. This fellowship supports investigation of a third genetic locus, *rok-1* (for **r**egulator **o**f **k**inase mediated signalling), which is thought to negatively regulate LET-23 mediated signalling.

During normal development, the gonadal anchor cell induces 3 of 6 vulval precursor cells (VPCs) to divide and differentiate into vulval tissue. The inducing signal is encoded by the *lin-3* gene, a member of the EGF family of growth factors. The response to this signal is believed to be mediated by *let-23*, *sem-5*, an SH2/SH3 'adaptor' homologous to the mammalian GRB2 protein, *let-60 ras*, *lin-45 raf*, *mek-5* and *sur-1/mpk-1*. Hypomorphic mutations in any of these genes results in a vulvaless phenotype. *sli-1* was isolated as an extragenic suppressor of hypomorphic mutations in *let-23* and is found to be unable to suppress *let-23* null alleles. In the absence of other mutations, mutations at the *sli-1* locus are silent. *rok-1*(*sy247*) was identified in a screen in which *sli-1*(*sy143*) animals were mutagenized with EMS and F2 animals with greater than wild-type vulval differentiation selected.

rok-1(*sy247*) is a temperature sensitive mutation. At 20°C, the *rok-1*(*sy247*) mutation is silent in the absence of other mutations, however at 25°C, *rok-1*(*sy247*) animals are occasionally hyperinduced with an average level of induction at 3.1 cells per animal. Further, at 20°C, *rok-1*(*sy247*); *sli-1*(*sy143*) animals have on average 3.8 VPCs induced whereas at 25°C this is increased to 4.5. Thus, *rok-1*(*sy247*) is unlikely to be a null mutation. However, deficiency analysis suggests it is a strong loss of function mutation. We have constructed animals that in addition to having the *rok-1*(*sy247*) mutation, are mutated in *lin-3*, *let-23, sem-5, let-341* and *let-60 ras*. (Table 4). The molecular identity of *let-341* is unknown but, by epistasis, acts downstream of *let-23* and upstream of *let-60* and therefore may encode a *sos* homologue. We have found that *rok-1*(*sy247*) is able to suppress mutations in *lin-3*, *let-23* and *sem-5*. However, *rok-1*(*sy247*) is unable to suppress a *let-23* null, *let-341* or *let-60 ras* (Table 4). Thus *rok-1* appears to act upstream of *let-60 ras* to negatively regulate *let-23* mediated signalling. The strong suppression of *sem-5*(*n1619*) is particularly notable and may indicate that *rok-1* acts at this step. Also notable is the relatively weak suppression of *let-23*(*sy97*) compared to that seen by *sli-1*. The *let-23*(*sy97*) mutation deletes the three potential tyrosines in the receptor's carboxy terminal tail which may be phosphorylated creating SH2 binding sites which match the consensus binding site for the SEM-5 SH2 domain.

We have cloned the *rok-1* locus and found it to encode a single transcript of 3.6 kilobase (based on library screening; Figure 3). Expression of this 3.6 kilobase cDNA from a heat shock promoter is able to rescue the defects associated with the *rok-1*(*sy247*) mutation. The predicted open reading frame of this transcript encodes a novel cytoplasmic tyrosine kinase of the structure:



Given that the genetic evidence suggests that *rok-1* acts in the *let-23 - let-60* pathway, we sought to determine whether ROK-1's SH3 domain or potential SH3 binding sites in the proline rich C terminal tail interact with SEM-5 or SOS. To do this, we have used the yeast two hybrid system. Since the *C. elegans* homologue of *sos* is not yet cloned we used *Drosophila* SOS. In these assays, we detect interaction between ROK-1's proline rich C terminal and the C terminal SH3 domain of SEM-5. We also detect interaction between *Drosophila* SOS and the C terminal SH3 domain

of SEM-5. However, we detect no interaction between ROK-1's SH3 domain and ROK-1's own proline rich tail or with SOS (Table 5). In addition, we also have failed to detect interaction between the N terminal SH3 domain of SEM-5 and ROK-1.

Thus, we have physical and genetic evidence that *rok-1* acts in the receptor tyrosine kinase - *ras* pathway. The *sem-5* mutation *n1619* results in the substitution of a proline for leucine in the N terminal SH3 domain of SEM-5 and produces a protein severely compromised in its functions. *rok-1(sy247)* allows this defective adaptor protein to signal effectively. The physical interaction between ROK-1 and SEM-5 utilizes the C terminal SH3 domain of SEM-5 and is therefore consistent with a model that this interaction leads to negative regulation. The relatively weak suppression of *let-23(sy97)* may indicate that negative regulation by *rok-1* requires the receptor's SEM-5 binding sites. This is also consistent with the model that negative regulation by *rok-1* is mediated through SEM-5. There are two obvious models of how the ROK-1:SEM-5 interaction could be used for negative regulation. First, this interaction itself could negatively regulate SEM-5 from binding SOS by competition. Alternatively, this interaction could be a mechanism of recruitment of ROK-1 into the complex which then leads to negative regulation by other means.

Table 4. Suppression by *rok-1(1f)* of vulval defects of loss-of-function pathway mutations. Double mutant strains were constructed by standard methods (Brenner, 1974; Ferguson and Horvitz, 1985; Huang and Sternberg, 1995). *data from Clark *et al.*, Nature, **356**, 340-344. †data from Sundaram and Han, Cell, **83**, 889-901.

	Average number of VPCs induced	
Genotype	***rok-1(+)***	***rok-1(sy247)***
+/+	3.0 (many)	**3.0** (many)
lin-3(n378)	**0.8** (29)	**2.85** (20)
lin-2(e1309)	**0.5** (20)	**1.4** (12)
lin-7(e1413)	**0.9** (20)	**3.2** (19)
lin-10(e1439)	**0.5** (20)	**1.9** (15)
let-23(sy1)	**0.8** (20)	**2.2** (21)
let-23(sy97)	**0.01** (>20)	**0.19** (21)
let-23(sy97);sli-1(sy143)	**2.87** (131)	**3.0** (20)
let-23(sa62)	**4.2** (147)	**4.1** (20)
sem-5(n2019)	**0.5** (20)	**1.28** (20)
sem-5(n1619)	**0.25** (8*)	**2.89** (23)
let-341(n2023)	**2.15** (20)	**2.36** (21)
let-60(n2035)	**0.016** (62)	**0.0** (20)
let-60(n1046)	**4.17** (240†)	**5.45** (20)

Table 5. Two-hybrid analysis.
GBT-9 encodes the GAL-4 DNA binding domain fused in frame with test protein. GAD-424 encodes the GAL-4 transactivation domain fused in frame with test protein. (-) indicates no fusion protein and thus a control construct. + indicates growth of yeast strain and hence protein-protein interaction; - indicates no growth and hence no interaction.

Test constructs:	INTERACTION?
GBT-9 (-) / GAD-424 (-)	-
GBT--1Δ1-834 / GAD-424 (-)	-
GBT-9:rok-1Δ1-834 / GAD-424:sem-5 N terminal SH3	-
GBT-9:rok-1Δ1-834 / GAD-424:sem-5 C terminal SH3	+++
GBT-9:rok-1Δ1-834 / GAD-424:rok-1 SH3	-
GBT-9 (-) / GAD-424:sem-5 N terminal SH3	-
GBT-9 (-) / GAD-424:sem-5 C terminal SH3	-
GBT-9 (-) / GAD-424:rok-1 SH3	-
GBT-9:rok-1 SH3 / GAD-424 (-)	-
GBT-9:rok-1 SH3 / GAD-424: DsosΔ1-1185	-
GBT-9 (-) / GAD-424: DsosΔ1-1185	-
GBT-9:sem-5 C terminal SH3 / GAD-424 (-)	-
GBT-9:sem-5 C terminal SH3 / GAD-424: DsosΔ1-1185	+++++

Technical details: Lethality was measured using the parental strain *unc-24(e138) let-60(n2035) rok-1(sy247) unc-31(e169) dpy-20(e1282) rok-1(sy247).*

Table 6. *let-60 (n2035)* F1 homozygote survival in both *rok-1(+)* and *rok-1(sy247)* backgrounds at 20°C. [1]Larvae indicates the total number of surviving F1 progeny. [2]Dpy indicates number of F1 progeny which segregate as Dpy-20. [3]Unc indicates the number of F1 progeny which segregate as Unc-24. All numbers are averaged over n parents used. The survival of *let-60(rf)* F1 homozygotes is represented as a percentage in bold in parentheses. It is calculated from the ratio of Unc to Dpy F1 progeny. *e138* is an allele of *unc-24*. *e1282* is an allele of *dpy-20*.

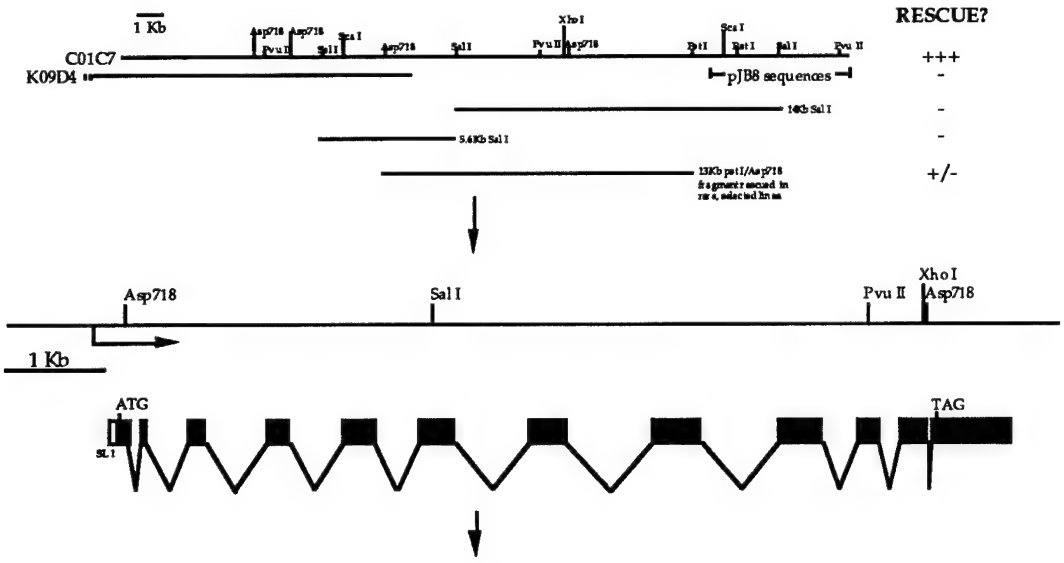
	parental *rok-1* genotype	
parental *let-60* genotype	***rok-1 (+)***	***rok-1 (sy247)***
	[1]Larvae: 129.0	
e138 n2035 / e1282	[2]Dpy: 35.4	Dpy: 386
	[3]Unc: 7.2 **(20 %)**	Unc: 289 **(74.9 %)**
	n=5	

Strains and general methods.

Methods for culturing and handling the nematode and general genetic methods were described by Brenner (Brenner, 1974). All genetic experiments were performed at 20 °C except when specified. The standard strain N2 was from Brenner (1974).

We observed the extent of vulval differentiation using Nomarski optics as described in (Han and Sternberg, 1990). We observed the vulva differentiation of the animals in their L3 molt stage, when the induced VPCs should have divided twice to generate four daughter cells and uninduced VPCs have divided once to generate two daughter cells. The average number of induced VPCs were calculated as the total number of induced VPCs divided by the number of animals observed. The average number of induced VPCs is in wild-type animals is three, and that in Hin animals is greater than three.

To ablate the anchor cell, we performed microsurgery using a laser microbeam. The procedure for microsurgery was described in (Avery and Horvitz, 1987, Sulston and White, 1980). We ablated the Z1, Z2, Z3, and Z4 cells, which include the precursors of the germ line, the gonad and the anchor cell, in the very early L1 stage animals. When the ablated animals are in their L3 molt, we observed their vulval differentiation using Nomarski optics as described above.



3.6 Kb cDNA which when placed under the control of a heat shock promoter creates hsp.rok-1 cDNA Stably transformed lines are rescued when heat-shocked at the appropriate time.

Figure 3. Molecular cloning of *rok-1*. A map of the rescuing cosmid C01C7 is shown above plasmid subclones. The 3 kb minimal rescuing genomic clone is enlarged and the relationship between it and the intron exon structure of rok-1 indicated. Injection method of Mello et al. (1991). **Injection protocol:** *rok-1(sy247)* is a temperature sensitive mutation: at 20°C 18/38 (47%) *dpy-20, rok-1(sy247); sli-1* animals are multivulva, while at 25°C 30/34 (88%) *dpy-20, rok-1(sy247); sli-1* animals are multivulva. *dpy-20(e1282), rok-1(sy247); sli-1(sy143)* animals were injected with 7.5 ng/ml test cosmid, 15ng/ml pMH86 (rescues *dpy-20;* Han and Sternberg, 1990) made up to a final concentration of 150 ng/ml with pBluescript and incubated at 25°C. Control data: *rok-1(sy247); sli-1(sy143)* animals at 25°C are 11/14 (79%) multivulva and *sli-1(sy143)* animals at 25°C are 0/16 (0%) multivulva.

4. Identification and cloning of additional negative regulators

We have devised and initiated a new screen for additional genes that interact with *sli-1.* While mutations in a single negative regulator gene causes little or no vulval phenotype, in general, any combination of two or more of these mutants causes a synthetic multi-vulva (Muv) phenotype. A general strategy for identifying new negative regulators of vulval induction has involved the mutagenesis of a single mutant, such as *sli-1*, and screening for multivulva animals; this works but has a high background of *lin-2, 7* and *10* mutations. We have taken a novel approach to perform a saturation screen for negative regulators of vulval induction while reducing the frequency of *lin-2, lin-7* and *lin-10* mutations. *lin-2, lin-7*, and *lin-10* activity is required in the vulva precursor cells for proper processing of the anchor cell signal. It has recently been reported that overexpression of the receptor, *let-23*, rescues the vulvaless phenotype of *lin-2* and *lin-7* mutations (Simske et al., 1996). We hypothesized that introduction of a high copy *let-23* transgene into a *sli-1* background would eliminate or reduce the frequency of *lin-2, lin-7*, and *lin-10* mutations in a screen for Muv animals in a *sli-1* background. We have shown that

lin-2; sli-1 is not Muv in a *let-23* overexpression background. Further, as a positive control, we have shown that *rok-1; sli-1* is Muv in a *let-23* overexpression background. *lin-7; sli-1* and *lin-10; sli-1* are currently being built in a *let-23* overexpression background. These data are summarized in Table 7.

Table 7. Vulval induction in strains overexpressing LET-23. *syIs31* is an integrated transgene containing *dpy-20(+)* DNA as a marker and *let-23(+)* to overexpress LET-23. n.d., not determined.

Genotype	Vulval Induction (20° C)	% > 3.0 VPCs vulval
dpy-20; sli-1; syIs31	3.03 (n=20)	5
sli-1 lin-2	4.3 (n=21)	n.d.
dpy-20; sli-1 unc-1 lin-2; syIs31	3.0 (n=20)	0
rok-1; sli-1	3.8 (n=22)	64
dpy-20 rok-1 unc-31; sli-1; syIs31	3.34 (n=20)	45
dpy-20; rok-1; sli-1	n.d.	47 (n=39)
lin-7; sli-1	3.3 (n=20)	n.d.

An initial screen of approximately 10,000 gametes was performed (Table 8). Two Muv mutants were isolated, *sy443* and *sy452*. Both alleles show a Muv penetrance of about 60%. It has been determined that *sy443* is *sli-1(sy143)* independent, while *sy452* is *sli-1(sy143)* dependent (i.e. *sy452* is only Muv when *sli-1(sy143)* is present). Both alleles are in the process of being mapped. We will extend this work by saturating this screen for all negative regulators of this type. Novel negative regulators will then be characterized as they relate to other components of the *let-23* mediated signal transduction pathway.

Table 8. Comparison of old and new screens for mutations that synergize with *sli-1(1f)*

	Old Screen [*sli-1*]	New Screen *sli-1; ⇑let-23 (+)*
number gametes screened	20,000	10,000
#*sli-1*-dependent mutations recovered	3	1
#*sli-1*-independent mutations recovered	14	1
Freq. of good mutants	1/6700	1/10000
Ratio of Good/unwanted	3/14	1/1

nrl genes

We discovered three new negative regulators, *nrl-1, nrl-2 and nn-3,* as extragenic suppressor of the vulvaless phenotype of *lin-3*, which encodes the peptide ligand for LET-23. The *nrl-1* mutation suppresses the vulvaless and male spicule defects of *lin-3* mutations but not its lethality. *nrl-1* maps to chromosome IV in a region suitable for molecular cloning. *nrl-1* acts at or prior to LET-23. *nrl-2* acts approximately between LET-60 RAS and LIN-45 Raf. *nrl-3* acts downstream of MAP kinase at the level of transcriptional regulators.

5. Gene interactions

To help elucidate the roles of the negative regulators in signal transduction, we will are using pathway analysis *in vivo*. *sli-1*, *rok-1* and *unc-101* are in distinct pathways because double mutants display more severe phenotypes than the single mutant. Similarly, we demonstrated that a *rok-1; rok-2* double mutant is more severe than either single mutant. *nn-3* synergizes with *sli-1, rok-1* and *sli-2* (Table 9). *nrl-2* synergizes weakly with *rok-1*. While *sli-1* and *rok-1* display synthetic lethality (Table 10), *rok-2(sy317)* does not: thus *rok-2* is not redundant with *rok-1* for viability.

Table 9. Summary of interactions among negative regulatory mutants. WT, Double mutant is wild-type; Muv, double mutant is at least partially multivulva; nd, not yet determined.

	lin-15B	***unc-101***	***sli-1***	***sli-2***	***rok-1***	***rok-2***
lin-15A	Muv	WT	Muv [25°]	nd	Muv	nd
lin-15B		WT	WT	nd	WT	nd
unc-101			Muv	nd	Muv	nd
sli-1				WT	Muv	Muv
sli-2					nd	nd
rok-1						Muv
nrl-3	nd	nd	nd	nd	nd	nd

Table 10. Viability in strains defective in *rok-1*, *"rok-2(sy317)"* and *sli-1*. Measurements were performed using well nourished parents who had been at the test temperature for at least two generations.

	Viability	
Strain	**20°C**	**25°C**
N2	72/72 (**100%**)	166/182 (**91%**)
rok-1(sy247)	39/40 (**97.5%**)	47/49 (**96%**)
rok-1(sy247); sli-1(sy143)	218/254 (**86.2%**)	51/105 (**48.6%**)
sy317; rok-1(sy247)	100/114 (**87.7%**)	109/171 (**64%**)
sy317; sli-1(sy143)	262/284 (**92.2%**)	201/250 (**80.4%**)
sy317; rok-1(sy247) unc-31; sli-1(sy143)	61/70 (**87.1%**)	55/119 (**46.2**)

LET-23 Y1225 Negative Regulator

In the course of a different project (USPHS HD-23690) we have found that a tyrosine in the cytoplasmic tail of LET-23, Y1225, acts negatively on LET-60 Ras activation by the receptor, (G. Lesa and P. Sternberg, submitted). Since a receptor with Y1225 but no positively-acting carboxyl terminal tyrosines can inhibit in trans, it is likely that Y1225 defines a protein binding site, or at the least, a region of LET-23 whose conformation is necessary for binding of a protein that acts negatively. We therefore have begun to test whether any of the known negative regulators require this site for their action. We test each in two ways.

First, we ask whether mutational inactivation of a negative regulator relieves the inhibition by Y1225, animals homozygous for a *let-23* null mutation (*sy17*) are inviable. Introduction of a wild-type *let-23* genomic clone rescues almost completely this inviability. Introduction of a mutant *let-23* clone lacking all carboxyl terminal tyrosines (mutated to phenylalanine) rescues viability to 10% of wild-type. Presence of Y1225 abolishes this rescue. We can thus ask whether mutational inactivation of a negative regulator restores rescue, indicating that the negative effect of Y1225 requires that negative regulator. We have constructed a recipient strain *let-23(syl7) unc-4(e120)/mnC1{dpy-10 unc-52}; dpy-20/dpy-20; sli-1/sli-1.* This strain allows survival of a Y1225 construct, suggesting that Y1225 requires SLI-1 for its effect.

A second test of the relationship between Y1225 and the known negative regulators is whether an intact LET-23 lacking only Y1225 can synergize with a mutation in a known negative regulator. Synergy would indicate that Y1225 does not act via that particular regulator, by analogy with the synergistic effect of, for example, a *sli-1* and a *rok-1* mutation. Lack of Y1225 did not synergize with a *rok-1* mutation to activate the LET-60 Ras pathway and cause a multivulva phenotype. This latter result is not consistent with the simplest models, and we will await analysis of other negative regulators by these two assays before concluding whether Y1225 acts via SLI-1.

Conclusions

•**Mechanistic studies of SLI-1.** The action of SLI-1 has been further localized to a role in modulating Ras activation. Additional mechanistic studies will now be aimed at delineating its mode of action.

Given the now intense interest in the cbl/SLI-1 family of proteins, basic molecular genetic analysis of the *C. elegans sli-1* gene will be pursued. To help understand the structure function relationship in this family, additional mutant alleles of *sli-1* will be sequenced. Also, systematic site-directed mutagensis studies will be initiated assaying the function of *sli-1* in transgenic nematodes. The results of these studies will be communicated directly to those researchers studying human cbl proteins to help them design their experiments.

•***rok-1* encodes a novel protein tyrosine kinase that interacts with the adaptor SEM-5**. The *C. elegans rok-1* locus, encoding a negative regulator of the LET-23 tyrosine kinase/LET-60 Ras pathway has been molecularly cloned and its DNA sequence will be determined over the next several months. Extensive genetic and phenotype characterization of *rok-1* has been carried out. Besides revealing much about the molecular identify of this negative regulator, knowledge of the sequence will allow human homologs to be identified.

•**New negative regulators.** New potential negative regulators have been identified. These loci will be genetically mapped and cloned. With >50% of the *C. elegans* genome sequenced, all loci can be cloned fairly rapidly.

Progress by task as per original Statement of Work:

A brief description of progress on each task for the first year is listed.

Task 1A. Determine whether SLI-1 truncation decreases or increases activity of the protein as assayed in transgenic animals [months 1-6]. a. construct truncated forms by site-directed mutagenesis. •Completed

Task 1B. Determine expression of SLI-1 in *C. elegans*. •Completed.

Task 1C. •[behind schedule]

Task 1D. •[behind schedule]

Task 2. Molecular cloning of SLI-2 from *C. elegans*. a. Correlation of genetic and physical mapping will identify a region of genomic DNA including the *sli-2* locus and less than 150 kilobases of DNA. [months 1-6]. •*sli-2* has been mapped to a region of 150 kb [on schedule] **b. Microinjection of cloned genomic DNA will be used to define the smallest region of DNA that provides *sli-2* function in transgenic animals [months 7-12].** [behind schedule]

Task 3. Molecular cloning of ROK-1 from *C. elegans*. *rok-1* cloned and sequenced. A candidate human homolog identified by homology. Mechanistic studies of ROK-1 initiated. [on schedule]

Task 4. Identification by genetic screens of new loci.

a. Screen for new mutations, carry out screens in parallel. [months 1-24]. •A modified screen for *sli-1* synthetics has been devised, to lower the background of *lin-2*, *lin-7* and *lin-10* mutants. [on schedule]

b. Genetic mapping and complementation of new mutations, parallel experiments [months 3-27] •Three other loci, have been identified and mapped to linkage groups. [on schedule]

Task 5. Examination interactions of genes in vivo [1 month, part-time/experiment] ongoing •[on schedule]

Task 6. Human homologs. ROK-1 structure will permit an effective search for human homologs. •[on schedule]

Task 7. Introduction of c-cbl cDNA into transgenic nematodes. a. **Construct *sli-1/c-cbl* hybrid genes [months 6-7]. b. Examine phenotypes of transgenic animals [months 8-14].** •c-cbl does not work in *C. elegans*; chimaeras under construction. *sli-1* minigene construction completed. [on schedule]

The research team is now in high gear. Graduate students Christopher Lacenere [new screens] and Charles Yoon [*sli-1* and *sli-2*], postdoctoral fellow Neil Hopper [*rok-1* and *rok-2*], and two research assistants [helping on the molecular cloning of *sli-2* and *rok-2*] are engaged full-time on the project with part-time help from other postdoctoral fellows and graduate students on gene interactions, new genes and molecular cloning experiments.

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